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# Induction of Glomerular Heparanase Expression in Rats with Adriamycin Nephropathy Is Regulated by Reactive Oxygen Species and the Renin-Angiotensin System

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Heparan sulfate (HS) in the glomerular basement membrane (GBM) is important for regulation of the charge-dependent permeability. Heparanase has been implicated in HS degradation in several proteinuric diseases. This study analyzed the role of heparanase in HS degradation in Adriamycin nephropathy (AN), a model of chronic proteinuria-induced renal damage. Expression of heparanase, HS, and the core protein of agrin (to which HS is attached) was determined on kidney sections from rats with AN in different experiments. First, expression was examined in a model of unilateral AN in a time-course study at 6-wk intervals until week 30. Second, rats were treated with the hydroxyl radical scavenger dimethylthiourea (DMTU) during bilateral AN induction. Finally, 6 wk after AN induction, rats were treated with angiotensin II receptor type 1 antagonist (AT1A) or vehicle for 2 wk. Heparanase expression was increased in glomeruli of rats with AN, which correlated with HS reduction at all time points and in all experiments. Treatment with DMTU prevented the increased heparanase expression, the loss of GBM HS, and reduced albuminuria. Finally, treatment of established proteinuria with AT1A significantly reduced heparanase expression and restored glomerular HS. In conclusion, an association between heparanase expression and reduction of glomerular HS in AN was observed. The effects of DMTU suggest a role for reactive oxygen species in upregulation of heparanase. Antiproteinuric treatment by AT1A decreased heparanase expression and restored HS expression. These results suggest involvement of radicals and angiotensin II in the modulation of GBM permeability through HS and heparanase expression.

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The glomerular basement membrane (GBM) consists of many extracellular matrix proteins, including heparan sulfate (HS) proteoglycans, mainly agrin. Loss of negatively charged HS molecules results in an altered charge-dependent permeability of the GBM (1). The importance of HS in the charge-dependent permeability of the GBM has been demonstrated in several studies. First, digestion of HS by heparitinase resulted in increased permeability of the GBM for ferritin and albumin (2,3). Second, injection of a specific mAb against HS caused massive albuminuria in rats (4). Third, loss of anionic HS has been reported in several human and experimental glomerulopathies, which was inversely correlated with the degree of proteinuria (5,6). It is suggested that proteinuria-

induced renal damage is associated with loss of glomerular HS in diabetic and nondiabetic renal disease (7–9).

HS loss in proteinuric renal disease can be attributed to several mechanisms (reviewed in reference [1]), such as depolymerization of HS by reactive oxygen species (ROS), masking of HS by nucleosome/Ig complexes, reduction of both HS production and sulfation as a result of hyperglycemia, complement-mediated cleavage of HS, and proteolytic cleavage of the HS attachment site on the core protein by enzymes.

Heparanase is an endo- $\beta$ (1,4)-D-glucuronidase that is involved in the cleavage of HS and hence is associated with extracellular matrix degradation and tissue remodeling (10–13). A recent study in patients with diabetic nephropathy suggested that loss of HS in the GBM is attributable to accelerated HS degradation by increased heparanase expression (14). Studies in experimental renal diseases (passive Heymann nephritis, puromycin aminonucleoside nephrosis, and anti-GBM nephritis) suggest that heparanase also may be involved in nondiabetic proteinuric disease (15–18).

In chronic proteinuric renal diseases, blockade of the renin-angiotensin system (RAS) reduces proteinuria and thereby reduces progressive renal function loss. In Adriamycin nephrop-

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athy (AN), a model of chronic proteinuric renal damage (19), we previously reported a decrease of HS expression, which was caused partially by hydroxyl radicals (7). Furthermore, we found that RAS blockade protects against loss of HS in established AN (9). However, whether changes in heparanase may be involved in the decrease of HS expression in AN and/or the effects of RAS blockade is unknown. Therefore, in this study we first determined the time course of heparanase and HS expression in relation to the development of proteinuria and renal structural damage in AN. For this purpose, we used the unilateral variant of the AN model to allow good resolution over time. Next, the effect of scavenging of ROS during the period of induction of AN on heparanase and HS expression and on proteinuria was studied. Finally, we evaluated the potential of antiproteinuric treatment with an angiotensin I receptor antagonist (AT1A) to restore the disbalance between heparanase and HS in established AN with persistent proteinuria.

## Materials and Methods

### Animals and Experimental Design

Male Wistar rats were housed in a temperature-controlled room with a 12-h light-dark cycle and with free access to food and water. Twenty-four-hour urine samples were collected every 2 wk in metabolic cages, with measurement of water and food intake. Surgical procedures took place under isoflurane anesthesia in N<sub>2</sub>O/O<sub>2</sub> (1:2). Systolic BP was measured weekly by the tail-cuff method in conscious rats (20). At the end of the study, the abdominal aorta was cannulated, a 2-ml blood sample was taken, and kidneys were perfused *in situ* with saline and removed. Proteinuria was measured on a BNII third-generation nephelometer (Dade Behring, Mannheim, Germany) by using a 20% trichloroacetic acid solution. The Committees for Animal Experiments of the University of Groningen and the Radboud University Nijmegen, The Netherlands, approved all studies.

**Experiment 1.** Unilateral AN was induced by temporarily clipping the left renal artery and vein through a midline abdominal incision (20), followed by Adriamycin (1.5 mg/kg body wt) injection *via* the tail vein. After 12 min, when Adriamycin had been cleared from the circulation (19), the clamp was removed. For studying expression of hepara-

nase, HS and renal damage over time, eight rats (at each time point) were killed at weeks 6, 12, 18, 24, and 30. In this experiment, the right kidney—exposed to Adriamycin—was compared with the control (nonexposed, left) kidney. Previously, we showed that the clipped left kidney did not differ from healthy control kidneys, not even after 30 wk (21).

**Experiment 2.** Bilateral AN was induced by intravenous injection of 5 mg/kg body wt Adriamycin *via* the tail vein. One group of rats (*n* = 8) received an initial intraperitoneal injection of the hydroxyl scavenger dimethylthiourea (DMTU; 500 mg/kg body wt; Sigma, St. Louis, MO) 6 h before Adriamycin injection, followed by treatment with DMTU (1.25 mg/kg body wt) twice a day for 7 d. The control group (*n* = 7) received saline instead of DMTU. Rats were killed 4 wk after the injection of Adriamycin. Urine was collected at weeks 0 and 4 for determination of urinary albumin excretion.

**Experiment 3.** Bilateral AN was induced by intravenous injection of Adriamycin (2 mg/kg body wt) *via* the tail vein. At week 6, a renal biopsy was performed *via* a dorsolateral incision. After removal of a part of the lower pole from the left kidney, gel foam (Spongostan; Ferrosan, Copenhagen, Denmark) was applied for hemostasis. After recovery, rats were treated with the angiotensin II type 1 receptor antagonist (AT1A) L158,809 (150 mg/L drinking water; *n* = 20) or vehicle (*n* = 10). In previous experiments, the biopsy did not affect the course of renal damage (9,22). Treatment was continued until the rats were killed at week 8. Eight healthy rats that were killed at week 6 were used as time controls.

### Immunofluorescence Staining

To determine heparanase, HS, and agrin core protein expression, we performed indirect immunofluorescence staining on 2- $\mu$ m cryostat kidney sections. Tissue sections were fixed in 100% acetone for 10 min at 4°C, followed by 1 h of incubation of primary antibodies diluted in PBS that contained 1% BSA and 0.01% sodium azide at room temperature. After washing in PBS, the appropriate secondary antibodies were diluted in PBS and incubated for 1 h at room temperature, with 5% normal rat serum and 10% normal goat serum. Specific primary and secondary antibodies that were used are summarized in Table 1. Subsequently, the sections were washed in PBS and embedded in Vectashield mounting medium H-1000 (Vector Laboratories, Burlingame, CA).

Table 1. Antibodies used for immunofluorescence

Antibody	Specificity and Epitope	Dilution	Source	Secondary Antibody <sup>a</sup>
HPA1	Rabbit anti-heparanase: 50-kD/8-kD heterodimer	1:100	ProsPec-Tany Technogene, Rehovet, Israel	Goat anti-rabbit IgG Alexa 594 or 488
JM403	Mouse anti-rat HS: N-unsubstituted glucosamine unit	1:300	(4,41)	Goat anti-mouse IgM Alexa 488
MI91	Hamster anti-agrin core protein: N-terminus	1:800	(42)	Goat anti-hamster IgG Cy3
OX7	Mouse anti-Thy1.1: Mesangium cells	1:300	PharMingen, San Diego, CA	Goat anti-mouse IgG Alexa 488
G1D4	Mouse anti-synaptopodin: Podocyte cytoskeleton	1:1	Progen Biotechnik, Heidelberg, Germany	Goat anti-mouse IgG Alexa 488
300	Mouse anti-calbindin D-28k: Distal convoluted tubuli	1:300	Swant, Bellinzona, Switzerland	Goat anti-mouse IgG Alexa 488
AQP2	Guinea pig anti-aquaporin 2: Collecting duct	1:300	Kindly provided by Dr. P. Deen (43)	Goat anti-guinea pig IgG Alexa 488
C219	Mouse anti-P-glycoprotein: Proximal tubuli	1:20	Abcam, Cambridge, UK	Goat anti-mouse IgG Alexa 488

<sup>a</sup>Secondary Alexa antibodies were purchased from Invitrogen (Molecular Probes, Breda, The Netherlands) and were used in a dilution of 1:200; Cy3-labeled antibody was obtained from Jackson ImmunoResearch Laboratories (Cambridgeshire, UK) and used in a dilution of 1:800.

For investigation of which glomerular cell type was responsible for the expression of heparanase in AN, sections were double-stained with anti-heparanase and (1) anti-agrin, to distinguish between the outside of the GBM covered by podocytes and the inside with endothelial cells lining the GBM; (2) anti-synaptopodin, which stains the cytoskeleton of podocytes; and (3) anti-Thy1.1, which is a mesangial cell marker. For investigation of in which tubular cell type heparanase was expressed, double stainings were performed with anti-heparanase and (1) anti-aquaporin 2, a marker for collecting ducts; (2) anti-calbindin D-28k, a marker for distal convoluted tubuli; and (3) anti-P-glycoprotein, a marker for proximal tubuli (summarized in Table 1). Confocal laser scanning microscopy (Leica, Heidelberg, Germany) was used to evaluate the stainings.

### Quantification of Immunofluorescence Staining and Histochemistry

The kidney sections were randomly coded and evaluated by two independent observers on a Zeiss Axioskop microscope (equipped with an epi-illuminator). For agrin and HS expression, linearity of the GBM was scored at an arbitrary scale of 0 to 10 (0 = no staining, 1 = 10% linear GBM staining, etc., with a maximum score of 10 for 100% staining). Heparanase expression was scored from 0 to 5 arbitrary units for staining intensity. Expression was analyzed in 25 glomeruli per animal.

Focal glomerulosclerosis (FGS) was scored semiquantitatively on periodic acid-Schiff-stained paraffin sections (4  $\mu$ m), as described previously (23). FGS lesions were defined as glomerular areas with mesangial expansion and adhesion formation simultaneously present in one segment. Scoring was performed on a scale of 0 to 4 in 50 glomeruli per kidney moving from outer to inner cortex. The FGS scores presented in Tables 2 and 3 are the median FGS scores per 50 glomeruli per experimental animal group multiplied by 100 (score in arbitrary units from 0 to 400).

### Statistical Analyses

Data are expressed as median and 95% confidence intervals. Differences between groups were determined by Kruskal-Wallis and Mann-Whitney *U* tests. Linear regression was performed to detect whether proteinuria and heparanase and HS expression were associated. Analyses were performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL) and GraphPad Prism, version 4.0 software (GraphPad Software, Inc., San Diego, CA). Statistical significance was regarded at  $P < 0.05$ .

## Results

### Clinical and Morphologic Data

Data on proteinuria and BP for the time-course study (experiment 1) and the intervention study (experiment 3) are shown in Tables 2 and 3, respectively. In unilateral AN, proteinuria was increased at all time points and BP was normal. The score

for FGS was increased in the Adriamycin-exposed kidney compared with the nonexposed control kidney and progressed over time. When rats were treated with DMTU before induction of bilateral AN, albuminuria at week 4 was lower compared with that in saline-treated rats with AN (255 [185 to 276] versus 353 [274 to 436] mg/24 h;  $P < 0.05$ ). In the intervention study at week 6 (before treatment with AT1A started), proteinuria was markedly increased compared with healthy controls. Treatment during 2 wk with AT1A reduced proteinuria and BP. The score for FGS did not change during the 2 wk of treatment. In the vehicle-treated group, proteinuria and BP and FGS score remained stable.

### Heparanase Overexpression Is Associated with Reduced HS Expression in AN: Time Course

Glomerular HS and heparanase expression in the exposed and nonexposed kidney, assessed by indirect immunofluorescence staining on cryostat sections, is shown in Figure 1. HS showed a nice linear staining along the GBM in control kidneys (Figure 1A). However, in Adriamycin-exposed kidneys, the intensity of HS expression in the GBM was reduced, whereas agrin expression remained unaltered (Figure 1B). Semiquantitative analysis revealed that HS was significantly reduced in Adriamycin-exposed kidneys at all time points without changes over time, whereas agrin core protein expression did not differ between control and Adriamycin-exposed kidneys (Figure 2, A and B). Heparanase expression was markedly increased in all Adriamycin-exposed kidneys (Figure 1D) as compared with the control kidneys (Figure 1C), which was confirmed by semiquantitative analysis. Heparanase expression was increased significantly at all time points (Figure 2C) without changes over time. The reduction in glomerular HS expression significantly correlated with an increase in heparanase expression ( $R^2 = 0.34$ ,  $P < 0.001$ ; Figure 2D). Taken together, these results indicate that increased heparanase expression and loss of glomerular HS are early events in the time course of the Adriamycin-induced nephrotic syndrome.

### DMTU Reduces the Early Effect of Adriamycin on HS and Heparanase Expression

Because HS reduction in AN has been attributed to a depolymerization of HS by ROS (7), we studied whether the increased heparanase expression could be attributed to ROS by treating rats with the hydroxyl radical scavenger DMTU during disease induction by Adriamycin and the first week afterward.

Table 2. Characteristics of the time-course study in unilateral AN (experiment 1)<sup>a</sup>

	Week 6	Week 12	Week 18	Week 24	Week 30
Uprot (mg/24 h)	122 (72 to 367)	191 (142 to 414)	237 (183 to 395)	221 (70 to 351)	265 (159 to 459)
SBP (mmHg)	140 (112 to 146)	140 (122 to 166)	140 (122 to 156)	163 (120 to 183)	152 (138 to 190)
FGS control	0 (0 to 16)	2 (0 to 12)	8 (2 to 24)	19 (2 to 54)	24 (2 to 50)
FGS ADR	3 (0 to 16) <sup>b</sup>	20 (0 to 48) <sup>b</sup>	43 (10 to 78) <sup>b</sup>	45 (6 to 96) <sup>b</sup>	67 (38 to 148) <sup>b</sup>

<sup>a</sup>ADR, Adriamycin-exposed kidney; AN, Adriamycin nephropathy; FGS, focal glomerulosclerosis; SBP, systolic BP; Uprot, proteinuria.

<sup>b</sup> $P < 0.05$  versus control (nonexposed kidneys).



Table 3. Characteristics of the intervention study in bilateral AN (experiment 3)<sup>a</sup>

	Vehicle		AT1 Antagonist		Controls
	Week 6	Week 8	Week 6	Week 8	Week 6
Uprot (mg/24 h)	777 (254 to 883) <sup>b</sup>	697 (257 to 834) <sup>b</sup>	680 (250 to 904) <sup>b</sup>	245 (33 to 638) <sup>c,d</sup>	13 (11 to 17)
SBP (mmHg)	149 (140 to 171)	144 (97 to 156)	143 (124 to 164)	113 (60 to 148) <sup>c,d</sup>	140 (131 to 161)
FGS score	20 (0 to 30) <sup>b</sup>	30 (2 to 52)	12 (0 to 97) <sup>b</sup>	16 (2 to 87)	0 (0 to 2)

<sup>a</sup>AT1, angiotensin II receptor type 1.  
<sup>b</sup>*P* < 0.05 versus controls.  
<sup>c</sup>*P* < 0.05 versus week 6.  
<sup>d</sup>*P* < 0.05 versus vehicle.

When the rats were killed, 3 wk later, a loss of HS was observed in the saline-treated rats that was partly prevented by the treatment with DMTU (Figure 3A), whereas expression of agrin was comparable in both groups (data not shown), which is in line with previous experiments (7). It is interesting that the glomerular heparanase expression in AN rats that were treated with the ROS scavenger DMTU was significantly lower compared with that in saline-treated AN rat (Figure 3B).

### Reduction of Heparanase and Increase in HS Expression after Treatment with AT1A

Next, we investigated whether, in established proteinuria, antiproteinuric treatment by AT1A can reduce glomerular heparanase expression along with restoration of HS expression in the GBM. HS (proteoglycans) and heparanase expression was determined in bilateral AN, before (week 6) and after 2 wk of treatment with AT1A or vehicle (week 8). Glomerular HS expression was decreased in all Adriamycin-exposed animals at week 6, and heparanase expression was markedly increased, which was comparable with the results observed in the unilateral model of AN (Figure 4). Treatment with AT1A significantly increased glomerular HS expression compared with the vehicle-treated animals (Figure 4A), which was, interestingly, accompanied by a significant reduction in heparanase (Figure 4B). However, AT1 receptor blockade did not completely restore HS expression to normal levels as observed in the healthy controls. For the individual Adriamycin animals (vehicle and AT1A at weeks 6 and 8), the reduction in glomerular HS expression was significantly correlated with increased heparanase expression ( $R^2 = 0.63$ ,  $P < 0.001$ ; Figure 4C).

### Heparanase Expression in Glomeruli and Tubuli

Heparanase expression in AN is located at the outer side of the GBM and is minimally expressed within the capillary loops (Figure 5, A through C). Co-localization with synaptopodin confirms that heparanase is expressed by the podocytes and not by mesangium cells, because no co-localization with anti-Thy1.1 could be observed.

Heparanase is expressed in tubuli of both normal and diseased animals. Tubular HS and heparanase expression was not altered by either Adriamycin injection or AT1A treatment compared with controls. Using specific tubular markers, we observed that heparanase is expressed mainly in proximal tubuli.

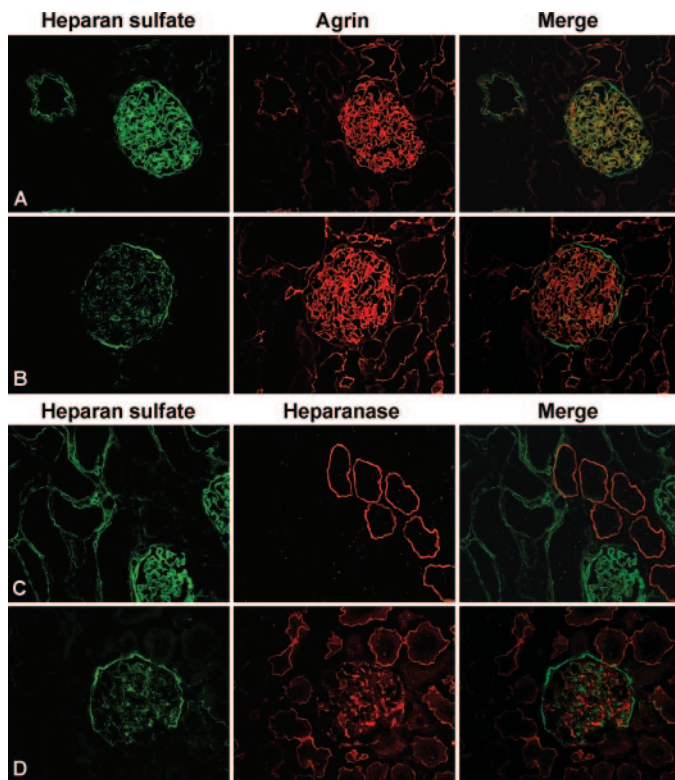
No expression was found in the distal convoluted tubuli or collecting ducts (Figure 5, D through F).

## Discussion

Our data demonstrate that glomerular heparanase expression is increased in Adriamycin-induced nephropathy and associated with a decreased HS expression in the GBM. These changes occur early after disease induction and remain stable during follow-up, whereas renal structural damage progresses. We previously found ROS to induce loss of glomerular HS (7); our data show that treatment with the ROS scavenger DMTU reduces heparanase expression compared with saline-treated Adriamycin rats, with subsequently a better preservation of glomerular HS expression and amelioration of albuminuria. Finally, glomerular heparanase expression in established AN was reversible by antiproteinuric treatment with RAS blockade, along with an increased glomerular HS expression.

In a previous study, we reported that by treatment with a ROS scavenger, HS expression was not completely restored and albuminuria was not completely prevented in AN (7), suggesting that additional mechanisms are involved in the reduction of HS in this model. Our study demonstrates that heparanase expression is increased in AN and correlated with the loss of HS in the GBM, suggesting that heparanase may play an important role in HS reduction. The increased expression of heparanase in AN is in line with the findings in puromycin aminonucleoside nephrosis, passive Heymann nephritis, and anti-GBM nephritis (15–18). The loss of glomerular HS also is in accordance with previous studies in proteinuric renal disease (7–9). Our data on a consistent association between HS and heparanase suggest that heparanase is an important factor involved in the breakdown of HS and thereby in the development of proteinuria. The pathogenic potential for heparanase in proteinuria is supported by a recent study showing that inhibition of heparanase prevented both proteinuria and loss of HS in passive Heymann nephritis (16). Moreover, heparin and heparin derivatives that inhibit heparanase have been shown to exert antiproteinuric effects in diabetic nephropathy, further supporting the impact of heparanase (24).

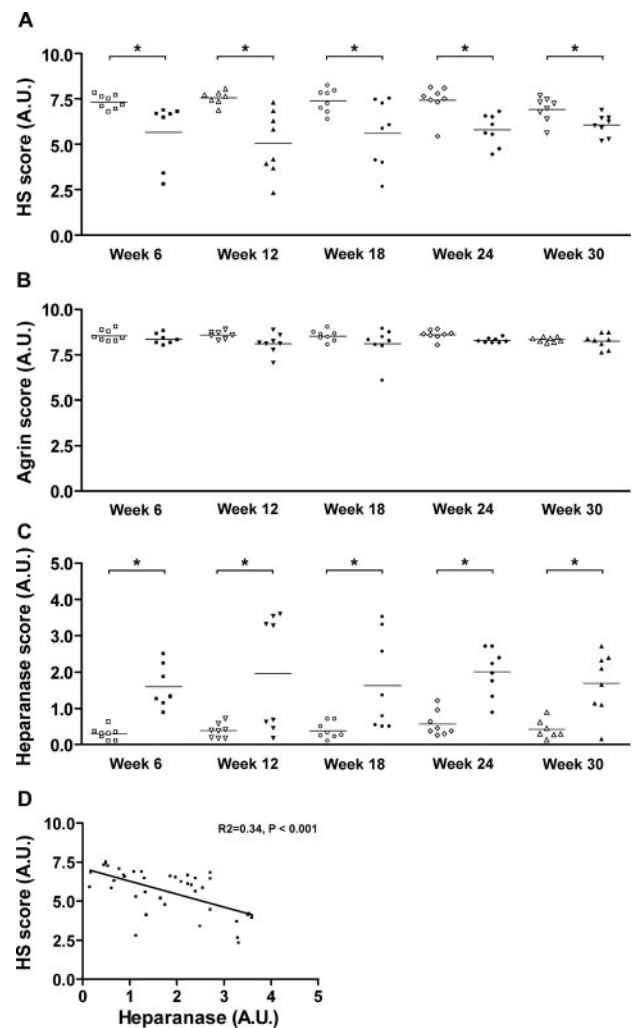
As mentioned before, ROS have already been demonstrated to be involved in the loss of HS in AN, which we explained by ROS-mediated depolymerization of HS (7). In this study, treat-



**Figure 1.** Heparan sulfate (HS; proteoglycans) and heparanase expression in Adriamycin nephropathy (AN) and control kidneys (indirect immunofluorescence staining). (A) JM403 staining for HS and MI91 staining for agrin core protein in a control kidney; a nice linear staining of the glomerular basement membrane (GBM) is observed for both HS and agrin. (B) HS and agrin staining in an Adriamycin-exposed kidney: Glomerular HS staining is decreased, whereas agrin staining remains linear. (C) HS and heparanase staining in a control kidney: Heparanase is present in the tubuli but absent in the glomerulus. (D) HS and heparanase expression in an Adriamycin-exposed kidney: A glomerulus with reduced HS expression and increased heparanase expression. Magnification,  $\times 40$ .

ment of AN with DMTU prevented the increase in heparanase expression and the loss of HS in the GBM. Whether ROS production in this model is persistent seems unlikely, because treatment with DMTU (6 h before and for only 1 wk after Adriamycin administration) partially prevents proteinuria, loss of HS, and heparanase overexpression at later stages.

Treatment with an angiotensin-converting enzyme (ACE) inhibitor or AT1A ameliorates proteinuria in both human and experimental renal diseases, including AN, and provides renoprotection (21,25–30). Antiproteinuric treatment with AT1A in this study led to a partially restored glomerular HS expression. This effect of AT1A on HS expression in AN is comparable with the effect of ACE inhibition that preserved glomerular HS expression in rats with AN (9). Along with the restored HS expression after treatment with AT1A, we observed a markedly reduced glomerular heparanase expression. *In vitro* experiments show that angiotensin II reduces HS expression in the extracellular matrix of human podocytes, raising the possibility of a direct effect (31). No



**Figure 2.** Semiquantitative analysis of glomerular HS, agrin, and heparanase expression in arbitrary units (A.U.) at different time points in unilateral AN (experiment 1). (A) HS expression in the GBM was significantly decreased in AN at all time points. (B) Agrin core protein expression did not differ between Adriamycin-exposed and control kidneys. (C) Glomerular heparanase expression was upregulated in AN at all time points. (D) Correlation between HS staining and heparanase expression in unilateral AN. Open symbols indicate score of nonexposed kidneys; filled symbols indicate score of Adriamycin-exposed kidneys.  $*P < 0.05$ .

data on intrarenal angiotensin II are available in AN; however, we have reported elevated intrarenal activity of ACE in AN, consistent with a local activation of RAS (32).

We used three different approaches to study the expression of glomerular heparanase and HS in AN. The first was a unilateral model with a relatively low (1.5 mg/kg body wt) Adriamycin dosage to allow good resolution over time. In the second study, the acute bilateral AN model (5 mg/kg body wt) with DMTU treatment was used with short-term follow-up. Finally, in the chronic bilateral AN model with AT1 intervention, we used 2 mg/kg body wt. Despite variations in the induction and severity of the model, the observed correlation between HS and heparanase was present consistently.

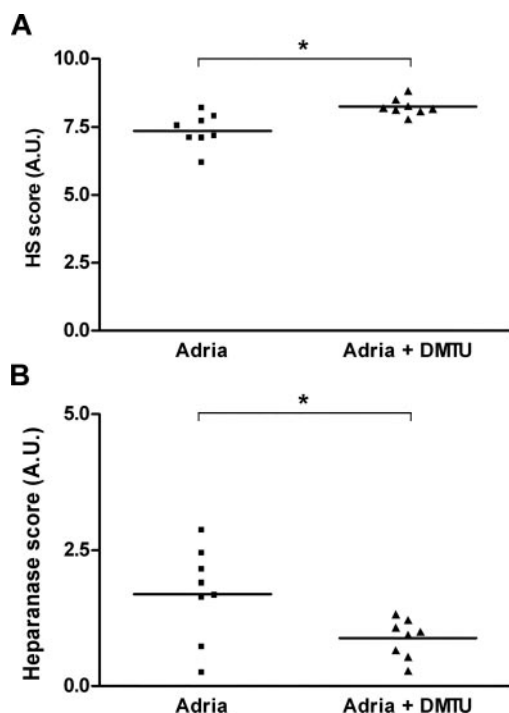


Figure 3. Effect of treatment with the hydroxyl radical scavenger dimethylthiourea (DMTU) before disease induction by Adriamycin and the first week afterward (experiment 2). (A) Decrease of HS expression in AN could be partly prevented by treatment with DMTU. (B) Heparanase expression was significantly lower after treatment with DMTU.  $*P < 0.05$ .

The changes in glomerular heparanase expression in the Adriamycin model and its reversibility by ROS scavenging or RAS blockade observed here theoretically could be due to different factors, namely (1) ROS, (2) angiotensin II, and/or (3) proteinuria. A single intrarenal administration of Adriamycin leads to a chain of reactions. Generation of ROS leads to injury of the podocytes, resulting in persistent proteinuria and increased production of (local) angiotensin II. All three elements (ROS, angiotensin II, and proteinuria), in our opinion, could be sequentially responsible for the persistence of heparanase overexpression. Although the generation of ROS can be induced by angiotensin II (33–35), ROS also are thought to be induced by Adriamycin *per se*. The beneficial effects of DMTU treatment during the induction phase of AN support a role for ROS in the induction of changed charge-selective properties of the GBM, as a result of both a direct effect of ROS on HS and ROS-induced heparanase expression. The effects of RAS blockade, showing reversibility of heparanase upregulation in established nephropathy, support a role for angiotensin II in heparanase expression. As to proteinuria, the time course study with early changes in heparanase and HS expression, which were stable during long-term follow-up despite prolonged exposure to proteinuria, suggests that increased heparanase and decreased HS expression are causal to proteinuria rather than a consequence. Furthermore, in a model of protein-overload nephropathy (36), we could not demonstrate a reduction in HS or an increase in heparanase expression (data not shown).

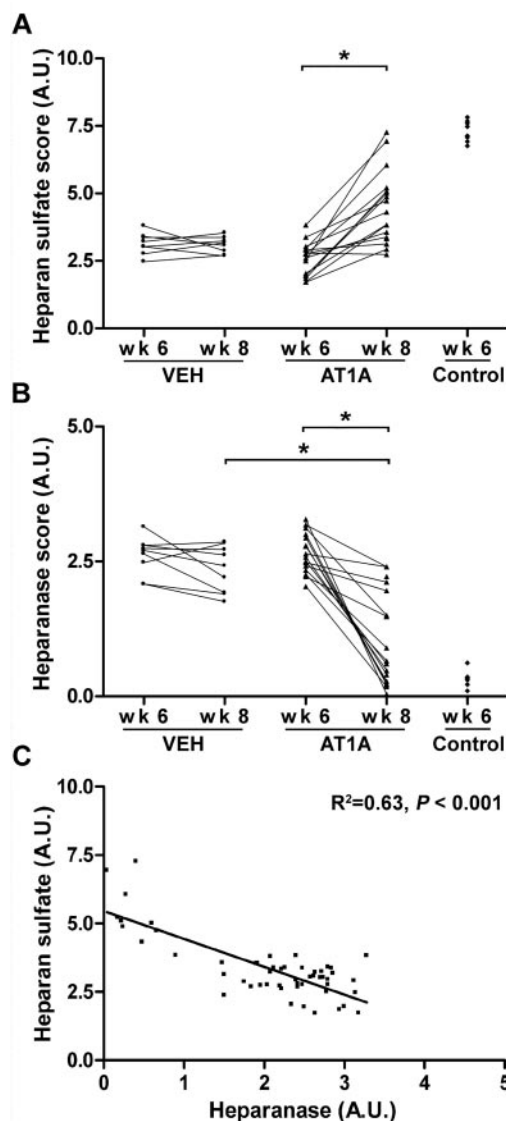
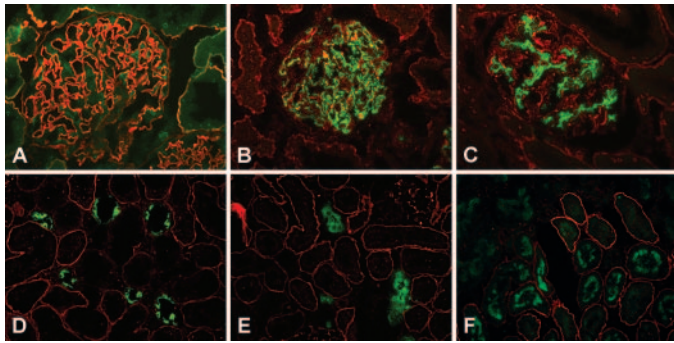


Figure 4. Effects of treatment with angiotensin type I receptor antagonist (AT1A) or vehicle (VEH) on heparanase and HS expression in bilateral AN (Experiment 3) compared with healthy control rats at week 6. Indirect immunofluorescence double staining was performed on kidney biopsies before (week 6) and after treatment (week 8). (A) Glomerular HS expression was reduced before treatment but restored after 2 wk of treatment with AT1A. Treatment with VEH did not restore HS expression. (B) After 2 wk of treatment with AT1A, heparanase expression decreased, and it did not change with VEH treatment. (C) Correlation between HS staining and heparanase expression in bilateral AN.  $*P < 0.05$ .

Our results suggest that both ROS and RAS are involved in upregulation of heparanase expression. Several studies have demonstrated that there is a link between ROS and RAS signaling. Angiotensin II has been shown to induce ROS production, whereas ROS mediates several effects of angiotensin II, such as on protein synthesis, cell hypertrophy, and vascular endothelial growth factor production. Scavengers of ROS ameliorate angiotensin II-induced or angiotensin II-mediated effects, whereas





**Figure 5.** Immunofluorescence double staining with anti-heparanase (green) and anti-agrin (red) (A), and with anti-heparanase (red) and anti-synaptopodin (B), or anti-Thy1.1 (C) (both in green). Glomerular heparanase is expressed mainly at the outside of the GBM, confirmed by co-localization with the podocyte marker synaptopodin. Minimal staining was observed in the capillary loops (according to the agrin/heparanase staining). No co-localization with mesangium cells was shown. For tubular heparanase expression, we used immunofluorescence double staining with anti-heparanase (red) and anti-calbindin D-28k (D), anti-aquaporin 2 (E), and anti-P-glycoprotein (F; all green). Tubular heparanase is expressed mainly in the proximal tubuli, because it co-localized with P-glycoprotein. No co-localization with the distal convoluting tubuli and collecting ducts was shown. Magnification,  $\times 63$ .

ACE inhibition or angiotensin II receptor blockade were able to reverse oxidative stress (37–40). Further studies would be needed to address the possible interactions between ROS and RAS in regulation of heparanase expression in AN.

## Conclusion

Heparanase plays an important role in the loss of HS in AN. Heparanase expression is increased early in the time course of AN and shows a clear association with the loss of HS in the GBM and proteinuria, suggesting that heparanase is an important mediator of loss of glomerular HS and development of proteinuria in AN. Scavenging of ROS prevented upregulation of heparanase and loss of HS. In addition to direct ROS-mediated depolymerization of HS, which we have shown previously *in vitro*, ROS indirectly may contribute to the HS loss in AN by upregulation of heparanase expression. It is feasible that in AN, both direct and indirect ROS-mediated mechanisms are operative. Reduction of heparanase and the subsequent restoration of glomerular HS contribute to the beneficial effects of RAS blockade. Our results suggest that both ROS and RAS play a role in heparanase induction and in the breakdown of HS in AN. However, the interplay between ROS and RAS in the induction of heparanase requires further investigation.

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